CLAIMS

[Claim(s)]

1. How to be adjustment method of biological activity liposome product containing biological activity amphiphilic compound relevant to liposome, and include the following processes. a). At least one lipid component connected by carrying out an electron pair share with water-soluble polymer is included. a process of mixing combination of lipid, and b -- a process of forming liposome stable in atomic arrangement from combination of this lipid. c) a process of obtaining liposome which has an average diameter of about 300 nm or less -- and -- d biological activity amphiphilic compound under conditions which are combined with liposome obtained from the above-mentioned process c, and activity conformation, A process of incubating liposome obtained from the above-mentioned process c with a biological activity amphiphilic compound.

2. Method given in the 1st paragraph of claim in which above-mentioned biological activity liposome product contains monolayer-like liposome.

3. Method given in the 1st paragraph of claim including process of forming multilocular liposome.

4. Method given in the 3rd paragraph of claim manufactured by attaining process at which above-mentioned multilocular liposome dries and rehydrates continuously liposome obtained at above-mentioned process c with biological activity peptide.

5. Method given in either of the 1st paragraph of claim to the 4th paragraph whose water-soluble above-mentioned polymer is polyethylene glycol (PEG).

6. Method given in the 1st paragraph of claim characterized by above-mentioned amphiphilic compound by having 1, alpha- beyond it, or pi-spiral donor in the biological activity conformation.

7. Method given in the 6th paragraph of claim whose above-mentioned compound is kind of growth hormone-releasing factor family of vasoactive intestinal PEPUCHI (VIP) / peptide.

8. Method given in the 7th paragraph of claim whose above-mentioned peptide is VIP.
9. Method given in the 1st paragraph of claim in which liposome obtained at above-mentioned process c has average diameter of 200 nm or less.
10. A method given in the 9th paragraph of a claim in which liposome obtained at the above-mentioned process c has an average diameter of 100 nm or less.
11. A method given in the 1st paragraph of a claim, the 8th paragraph, or the 9th paragraph acquired at the above-mentioned process c by extrusion in which the above-mentioned liposome forms liposome which has the selected average diameter.
12. A method given in the 1st paragraph of a claim, the 8th paragraph, or the 9th paragraph from which the above-mentioned liposome is obtained at the above-mentioned process c by size selection.
13. Combination of the above-mentioned lipid can set about combination of the further cholesterol (Chol). A method given in the 1st paragraph of a claim that consists of JISUTE aroyl phosphatidylethanolamine (PEG-DSPE), phosphatidylcholine (PC), and phosphatidylglycerol (PG) which are combined with PEG in electron pair share.
14. A method given in the 13th paragraph of a claim that combination of the above-mentioned lipid has combined with cholesterol in a mole ratio of PEG-DSPE:PC:PG:Chol which is 0.5:5:1:3.5.
15. An activity biologically liposome product manufactured by a method of a statement by either of the 1st paragraph of a claim to the 14th paragraph.
16. A constituent which contains a biological activity liposome product of a statement in the 15th paragraph of a claim that has the activity chosen from a group which the above-mentioned biological activity amphiphilic peptide becomes from antioxidation activity, crack recovery activity, wrinkles prevention activity, and aging prevention activity.
17. A constituent given in the 16th paragraph of a claim whose above-mentioned constituents are cosmetics.
18. A constituent given in the 16th paragraph of a claim whose above-mentioned constituent is an object for a therapy.
19. A constituent for medical examination which contains a liposome composition of a statement in the 15th paragraph of a claim, and contains a detection sign further.
20. A constituent for medical examination given in the 19th paragraph of a claim chosen from a group which the above-mentioned sign becomes from a compound which raises fluorescent labeling, a radioactive label, coloring matter, and magnetic resonance imaging.
21. A medical examination method including a process of preparing a constituent for medical examination of a statement in the 19th paragraph of a claim, a process of medicating a target tissue with an effective quantity on medical examination of this constituent, and a process of detecting ingestion of a constituent in a target tissue by sound wave reflection.

22. A constituent for medical examination given in the 21st paragraph of a claim chosen from a group which the above-mentioned sign becomes from a compound which raises fluorescent labeling, a radioactive label, coloring matter, and magnetic image resonance.
23. Oral controlled release pharmaceutical preparation for a therapy of an organ disease in which it is manufactured by a method given in the 7th paragraph of a claim, and the method includes a process of putting a biological activity liposome product into a capsule.
24. Oral controlled release pharmaceutical preparation given in the 23rd paragraph of a claim chosen from a group which the above-mentioned organ disease becomes from an inflammatory internal disease, chronic constipation, a leech SESHUSUPURUNGU disease, achalasia, a small-child nature thickening pyloric stenosis, and an ulcer.
25. A process of preparing a biological activity liposome product containing a biological activity amphiphilic compound relevant to liposome obtained by a method given in the 1st paragraph of a claim, It reaches. A method of medicating a target tissue with a biological activity amphiphilic compound including a process of medicating a target tissue with a quantity effective for a therapy of this liposome product.
26. A method given in the 25th paragraph of a claim characterized by having 1, alpha- beyond it, or a pi-spiral donor by amphiphilic compound in the above-mentioned biological activity conformation.
27. A method given in the 26th paragraph of a claim whose above-mentioned peptide is a kind of a growth hormone-releasing factor family of vasoactive intestinal peptide (VIP) / peptide.
28. A method given in the 27th paragraph of a claim whose above-mentioned peptide is VIP.
29. A method for holding an organ, tissue, or cell types of the body for transplantation of including a process of incubating an organ with a liposome composition manufactured by a method of a statement in the 8th paragraph of a claim.
30. Inflammation, hypertension, atherosclerosis, an inflammatory internal disease, chronic constipation, A use of a biological activity liposome product manufactured by the 1st paragraph of a claim including biological activity amphiphilic peptide for medicine for the therapy of a leech SESHUSUPURUNGU disease, achalasia, a small-child nature thickening pyloric stenosis, an ulcer, cell-growth promotion, and promotion of crack recovery of the body warehouse inside of a plane.
31. A use given in the 30th paragraph of a claim whose above-mentioned biological activity amphiphilic peptide is a kind of a growth hormone-releasing factor (GRF) family of vasoactive intestinal peptide (VIP) / peptide.
32. A use given in the 31st paragraph of a claim whose above-mentioned peptide is VIP.

[Translation done.]

EXAMPLE

(Comparison example)
The method of the advanced technology for the incorporation to VIP's liposome was reproduced for the purpose of providing the foundation for comparison with the method of this invention by this example. In old research, we decided [in / since bearing a certain role was suggested when VIP controlled stress of the vasomotion / in situ peripheral thin circulation] to examine first the VIP activity as a function of the carrier used in order to dissolve and send peptide. By enclosing VIP with whether the vasodilatation in peripheral thin circulation of a spontaneous hypertension hamster is caused further especially by VIP's local administration, and conventional single lamellae liposome, The first experiment was conducted in order to measure whether it can adjust any of the accepted reaction they are.
The normal-blood-pressure control group (n= 20) which the spontaneous hypertension hamster (n= 21) and the age, and the genetic factor of the male which matured suited was purchased from Canadian Hybrid Farms, Halls Harbor, NS, and Canada. As preparation, pentobarbital sodium (6 mg/100g weight) was injected intraperitoneally, and it applied to the animal at anesthesia, and tracheostomy was given in order to make spontaneous breathing easy. For one by one pouring to anesthesia (2 thru/or 4 mg/100g weight / time), Kanew ration was carried out to the left thigh vein. The catheter was inserted in the left femoral artery in order to record systemic arterial blood pressure and a heart rate. Body temperature was supervised so that 37 thru/or 38 ** might always be maintained [be / it / under / experimental period / letting it pass] using a heating pad.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
Material and a method for manufacturing an improvement liposome composition This application claims the right of priority over the U.S. provisional application 60th of application / No. 014 or 363 on March 28, 1996.
The background of an invention This invention generally relates to an activity constituent, the compound which has the both sides of amphiphilicity, i.e., a hydrophilic portion, and a hydrophobic part in more detail, and peptide biologically.
Especially this invention relates to the method of improving for delivery of amphiphilic peptide relevant to the liposome for the way in two ways of diagnosis and a therapy, and offer.
Activity biologically amphiphilic peptide which is a member of the family of peptide compounds including vasoactive intestinal peptide (vasoactive intestinal peptide) (VIP) and a growth hormone-releasing factor (GRF) observes especially by this invention. This invention relates to the improvement cure which sends peptide of a VIP/GRF peptide family to a target tissue through the member of peptide of a VIP/GRF peptide family, and the use of an improvement liposome composition which contains the activity analog biologically further especially.
VIP is a nucleotide which consists of 28 amino acid in which the extensive profile of a biological operation being shown and activating a multiplex signal introductory route is known. Refer to Said, PePtides 5(enlargement 1):149-150 (1984), Paul and Ehadi, Neurochem, Int, and 23:197-214 (1993). In Schiff-Edmundson ** of VIP as a double helix. Are on the confrontation of this whorl and it is shown clearly that nonpolar residue and polar residue have dissociated, Also when VIP is modeled as a bent alpha helix reported by Musso et al. and Biochemistry 27:8147-8181 (1988), this amphiphilic characteristic is clear. The tendency of a VIP similar pair which a whorl forms, and the relation between those biological activities are indicated to Bodan et al. and Bioorgan.Chem.3:133-140 (1974). The spectral characteristics of VIP in the inside of pure water are random coils, and change. However, an organic solvent and

leather ionicity lipid draw the whorl information in intramolecular. Robinson et al. and Biopolymers. 21:1217-1228(1983); -- refer to hamod et al., Biopolymers 22:1003-1021(1983); and Bodanszky et al., and Bioorganic Chem.3:133-140 (1974).
Having combined with the lipid bilayer the short peptide which can form an amphiphilic whorl, and having penetrated is known. Refer to Kaiser and Kezdy, Ann.Rev.Biophysical Chem.15:561-581 (1987) and Sanson, and Prog.Biophys.Molec.Biol.55:139-235 (1991). The appearance peptide model in which the example is indicated by DeGrado and Lear, and J.Am.Chem.Sac.107:7684-7689 (1985) (LKKLLKL-), And Watata and Gwozdzinski, and MERININ that is 26 residue bce BENOMU (venom) peptide currently indicated by Chem-Biol.Interactions 82:135-149 (1992) are drawn. Insertion of the arrangement of a peptide monomer parallel to the surface of the double layer mediated by electrostastic ****** between a polar amino acid and a phospholipid head group as a possible joint mechanism and the peptide aggregate to the selectively stable polar double layered core by canal operation is included. Refer to Sanson and Prog.Biophys.Molec.Biol.55:139-235 (1991).
VIP belongs to the family of family peptide and as other members, Peptide histidine isoleucine (PHI), peptide histidine methionine (PHM), growth hormone-releasing ** (GRF), hypophysis adenylate cyclase activity-ized peptide (PACAP), secretin, and glucagon are mentioned. Other members of a VIP/GRF peptide family and the amphiphilic whorl which can combine the activity analog with a lipid bilayer biologically can be formed like VIP. It is sure that a biological operation of the member of a VIP/GRF peptide family is mediated by the protein receptor and intracellular receptor which are revealed by the cell surface.
These days, it was shown that a calmodulin may be an intracellular receptor to VIP [Stallwood et al., J.Bio.Chem.267:19617-19621(1992); and Stallwood et al., FASEB J.7:1054 (1993)].

The main factor which restricts VIP's in vivo administration was the fall of the bioavailability in a target tissue because of the various conformation by which most was adopted as the denaturation of proteolysis nature, hydrolysis, and/or its peptide. Intracellular delivery of an itself [VIP] and/or VIP-calmodulin mixture avoids the demand to cell surface combination of peptide, and is conjectured to be able to promote a biological operation of peptide in this way. Since the lipid bilayer of liposome unites with the plasmlemma of a cell and the caught contents are sent to a cellular compartment, it is supplying the peptide made to reveal within liposome and on liposome, and, probably ******** delivery will be attained.
From the structure of liposome, and identification of the characteristic, although many proposal uses for the vesicle as a carrier for achieving the drugs delivery made into a target are drawn, it is not realizable for any of a large number most [the] various reasons. Most notably the remedial tendency use of conventional liposome, For the rapid incorporation to the reticuloendothelial system by a mononuclear phagocytic cell, What should be restricted

became clear [Greg0riadias and Ryman, Eur.J.Biochem.27:485-491(1972);Beaumler and Hwang, Biochem.Biophys.Acta 731:23-30 (1983)]. Although the incorporation by this specific-cells kind is advantageous under the limited conditions that a target cell or the organization itself is a part of reticuloendothelial system, Generally, phagocyte **** incorporation causes disassembly of the compound which should be sent, and a serious obstacle is encountered to this sending a compound to other cells or organization kinds.

In the trial to overcome, the problem which is inherent to liposome drug agent delivery research, Following incorporation of the liposome by a reticuloendothelial system, the identification of the compound considered to be re-emitted with HE in blood, Various compounds for increasing the stability of the liposome in the inside of the substituting method for intravenous administration of liposome, and a blood flow, For example, from the biophysics of [Kirby et al. turned to some trials including use of cholesterol, Biochem.J.186:591-598 (1980);Hwang, and liposome to thrapeutics, 109 - 156 pages of Ostro(piece) Marcel Decker.New York (1987); Beaumier et al. and Res.Comm.Chem.Pathol.Pharmacol.39:227-232 (1983)]. Furthermore, by other researches, in order to form the liposome double layer which imitated the double layer produced with the nature of an erythroid cell so that more closely, various lipid composition things were examined. These efforts the increase in the half-life of the liposome of a under [circulation]. It brought [Allen and Chonn, FEBS Lett.223:42-46 (1987);Gabizon and Papahajopoulos, Proc.Natl.Acad.Sci.(USA)85:6949-6953 (1988)].

PCT public presentation WO95/27496 and Gao et al., and Life Science 54:247-252 (1994) have indicated use of the liposome for VIP's delivery in comparison with delivery in solution. It became clear that enclosing VIP to the inside of liposome protected peptide from proteolysis denaturation, it promoted VIP's capability notably, and average arterial blood pressure was decreased compared with VIP in solution by a hypertension hamster. VIP who made liposome meet observed the lowest blood pressure in 5 minutes mostly after initial administration, and it became clear that average arterial blood pressure was remarkably reduced for about 12 minutes. This public presentation proved the combination to the liposome of VIP in the inside of solution, and penetration of peptide to a liposome double layer. It was guessed that the fall of peptide activity may be protected depending on stabilization of peptide to distribution of peptide to liposomal membrane and proteolysis or any of restricting peptide in activity conformation biologically combination of VIP to liposome is. Enclosure of VIP to the inside of that and liposome promoted the in vivo biological activity of peptide for which reason with the both sides of extending the effect in the blood pressure fell of a hypertension hamster, and increasing the size of the effect. Nevertheless, in the technical field concerned, offer of the further improving method [in / biologically / the delivery on the therapy of activity peptide and diagnosis] like VIP is still desired.

In this invention. The combination to water-soluble proteinic polymer. Lead and increase of the

half-life of the protein through which it circulates. [which notes observing -- Nucci et al. and Adv.Drug Del.Rev.6:133-151(1991); -- woodle et al. and Proc.Inter.Symp.Control.Rel.Bioact.Mater.17:77-78(1990)]. Such observation as an improvement drugs delivery system which minimized generating of rapid purification of the liposome from circulation notably, It led to development of the liposome (SSL) (known also as "PEG-liposome") stabilized in three dimensions [Lasic and Martin, Stealth Liposomes, CRC Press, Inc., BocaRaton, floor line (1995)]. SSL is the polymer and polymer covering liposome by which supply combination of the polyethylene glycol (PEG) is carried out preferably one of the phospholipid.

The outside of the vesicle double layer is provided with the hydrophilic cloud (cloud).

This three-dimensional barrier delays recognition by opsonin, SSL. From conventional liposome, for a long time, make it stop during circulation fairly and [Lasic and Martin, Stealth Liposomes, CRC Press, Inc.Boca Raton, floor line(1995);Woodle et al., and Biochem.Biophys.Acta. 1190:99-107(1994); -- Bedu Addo et al. and Pharm.Res.13:718-724 (1996)]. The pharmacologic effect of an enclosure reagent is reinforced as proved about some chemotherapeutic drugs and antiinfective drugs [Lasic and Martin, Stealth Liposomes, CRC Press, Inc., Boca Raton, floor line (1995)]. The factor from which the research in this field differs affects circulation half-life, [(9-12) Lasic which showed what the diameter of an average vesicle accompanied by PEG of the molecular weight of about 2,000 Da(s) should be [a thing] ideally less than 200 nm by 5% of concentration and Martin, Stealth Liposomes, CRC Press, Inc., and Boca. Raton, floor line(1995);Woodle et al., and Biochem.Biophys.Acta 1105:193-200 (1992); -- Lltzinger et al. -- Biochem.Biophys.Avta 1190:97-107(1994);Bedu. Addo et al., Pharm.Res.

13:718-724(1996)]. However, when the activity of a meeting compound may disappear with the SSL pharmaceutical preparation which has the desirable characteristic, preparation of SSL which has such physiological character and contains a bioactive compound does not exist without complication. Especially this is a case where a manifestation process is used for obtaining the liposome of the narrow small size of grain size distribution. For the reason which is not understood thoroughly, this extrusion process reduces substantially this liposome and the biological activity peptide compound which met. Therefore, although it is stable in three dimensions, it still asks for the improvement liposome composition which maintains the biological activity of a meeting peptide reagent.

In this invention, it takes notice of the indication of PCT public presentation WO93/20802 about multiplex lamellae liposome useful to the improvement in image quality of organic imaging by a sound wave (ultrasonic wave). This public presentation has indicated various liposome compositions with a range sizes [including the organization specific ligand like the drugs built into the lipid bilayer] of 0.8 thru/or 10 microns, in order to raise an antibody, an antibody

fragment, or an organization specific target. This oligo lamellae liposome is manufactured repeatedly [of freeze-drying and freezing-defrosting] by the improvement double becoming [turbid] method for manufacturing the double layer separated intrinsically. It is said that desirable liposome is a range 1.0 thru/or 3.0 microns in diameter. It was still much more difficult to manufacture liposome easily detectable with the conventional ultrasonic technique of less than the size of about 0.5 micron in this way. Therefore, it still asks for the improvement liposome composition which may be manufactured efficiently and has a mean grain size below about 0.5 micron. It is manufactured efficiently, and it is stable at in vivo, and still asks for the improvement liposome composition which provides more advanced solution in sound wave imaging.

In this way, in the technical field concerned, the necessity of providing the further method [therapeutic and] of improving a bioactive molecule in use of the liposome technology for diagnostic administration exists. Further especially, in the technical field concerned, in order to attain the effective medicine effect extended further, it is not limited, but it still asks for the method of improving for the administration of amphiphilic peptide including the member of a VIP/GRF peptide family.

Outline of an invention This invention relates to the improvement process of liposome and the activity biologically liposome products which were made to meet and which contain an activity amphiphilic compound biologically. This liposome formula of this invention is a form which brings about the improvement in the effect and temporal duration of biological effectiveness of meeting peptide, and sends and enhances the bioactive of activity peptide on this biology target. It is a result of the interaction of this compound and liposome the effect of this biological effectiveness, and increase of temporal duration sure with the form which this compound attains at least selectively.

There is activity or it is maintained by the conformation which has activity from the compound in aquosity environment.

In this way, although this invention is not limited, it overcomes the problem accompanying the conventional liposome formula like delivery of the compound by a reticuloendothelial system which incorporates and is in disassembly of a compound, or inactive conformation.

According to one mode of this invention, the combination of the lipid containing at least one sort of lipid composition things which carried out the covalent bond to a water solubility polymer is mixed, b) Form the liposome stabilized in three dimensions from the combination of this lipid, c) Obtain the liposome which has an average diameter below about 300 nm, and, subsequently to activity conformation, the liposome from the d process c under these conditions of the process c of being that are made to hold a liposome meeting, The process of the activity biologically liposome products in liposome and an association condition incubating with an activity amphiphilic compound biologically and which contain an activity amphiphilic

compound biologically is provided. According to another mode of this invention, generally, liposome products [activity / target / this / biology] are constituted from desirable single lamellae liposome by the therapy use. According to another mode of this invention, biologically activity liposome products, It comprises multiplex vesicle liposome and is manufactured by the method characterized by the further process of forming multiplex vesicle liposome by performing the process of drying and rehydrating the liposome preferably got by this biology target at the process c with the activity compound one by one. Although multiplex vesicle liposome, multiplex lamellae liposome, and oligo lamellae liposome are not "Mr. Onion" arrangement in spite of which meaning permitted it and reversely, it is interpreted as the liposome containing a time [which was shown by drawing 1] multiplex, and irregular inside "compartment." Although this multiplex vesicle liposome may be used for the therapy use of this invention, it is useful to the echo generating diagnostic method of this invention with which they show the surprising echo generating characteristic especially.
Like one mode of this invention, this liposome is liposome (SSL) which is manufactured from the combination of the lipid containing at least one sort of lipid components which carried out the covalent bond to water-soluble polymer and which was stabilized in three dimensions. This water-soluble polymer is a polyethylene glycol (PEG) preferably.
It serves to stabilize the obtained liposome in three dimensions to the incorporation by the component of a reticuloendothelial system.

Even if the method of this invention uses an activity amphiphilic compound biologically [any], it is useful, is in this liposomal lipid double layer and an association condition by that cause, or can be stabilized and maintained to the activity conformation in the letter bear of a meeting within this liposomal lipid duplex film. What the thing characterized by [those] having one or more alpha or pi whorl domains in activity conformation biologically especially polar residue, and nonpolar residue have divided into the opposite hand of this whorl as a desirable amphiphilic compound is contained. As a useful especially desirable amphiphilic compound, any member of the vasoactive intestinal peptide (VIP) / growth hormone-releasing factor (GRF) peptide family which contains the activity analog biologically is contained in this invention. As the mammals and a non-mammals VIP/GRF polypeptide family, Functional analog and peptide histidine isoleucine (PHI), peptide histidine methionine (PHM), the growth hormone-releasing factor (GRF), hypophysis adenylate cyclase activity-ized peptide (PACAP), secretin, and glucagon of VIP and GRF are mentioned. Other members and amphiphilic whorls which the hydrophobic domain and hydrophilic domain of the peptide can separate the activity analog, and the hydrophobic domain can combine with a lipid bilayer biologically of a VIP/GRF peptide family can be formed like VIP. This invention means the receptor antagonist which has the liposome manufactured by the method of this invention, and the enhanced bioactive in an

association condition. Especially desirable peptide for the use of this invention is VIP. Biologically [this invention], detecting the delivery which liposome products target-ized may be used for the various therapy uses and diagnostic use for which it asks so that it may be indicated to that activity peptide products send an activity compound biologically [a high level] or the following.

Furthermore, this invention provides the improvement sound wave diagnostic products in which they have a sound wave reflection property smaller at a pitch diameter than 1000 nm and more surprising than 300 nm irrespective of the fact of being even small. The result for which a diameter uses the liposome below 300 nm has especially surprising liposome in the light of the art of the technical field concerned which is a range 0.8 thru/or 3.0 microns in diameter. Especially this invention provides the manufacturing method and directions of the liposome for the multiplex lamellae diagnosis below about 1000 nm which has an about 500-nm average diameter for improvement imaging using sound wave reflective art especially. On these specifications, sound wave reflection, echo reflection, and ultrasonic imaging have the same meaning intrinsically, and are used. The method of this invention mixes the combination of the lipid which combined at least one sort of lipid components with water-soluble polymer, Liposome is formed and obtained from the mixed combination lipid, and it incubates with an activity ******** compound biologically, and is characterized by the process of forming the multiplex lamellae liposome which subsequently has an average diameter below about 1000 nm. According to the desirable example of this invention, this multiplex lamellae liposome is formed by enforcing a freeze drying method. In a desirable example, the liposome first formed from this lipid mixture has an average diameter below about 300 nm, and these liposome is obtained by extrusion molding by another example. Although the desirable multiplex lamellae liposome of this invention has an average diameter below about 800 nm, it has an average diameter below about 300 nm most preferably. In a desirable example, this water-soluble polymer is PEG. The thing which can form alpha or pi whorl domain and which is preferably chosen from the member of a VIP/GRF peptide family is contained in an activity compound biologically [this invention]. The most desirable activity biologically compound of this invention is VIP. although it is not planned to restrain which theory of those inventions, either -- water-soluble polymer like PEG -- multiplex lamellae liposome ******** -- it is believed by things that higher they of capability which are small sizes comparatively and which nevertheless reflect sound wave energy can be manufactured. Although he does not understand thoroughly why it becomes so, as one possibility existence of water-soluble polymer, It acts so that the wall of two or more liposome vesicles which build single multiplex vesicle liposome may be separated, and making sound radiation into the liposome which can be reflected better in this way is mentioned.

Furthermore, this invention provides an improvement sound wave diagnostic method, wherein

the small liposome for diagnosis of this invention medicates a target tissue with an activity amphiphile compound in an effective quantity diagnostically biologically and subsequently detects this liposome by in vivo using sound wave reflection. The desirable target tissue of this invention is a tumor. In one example, an activity compound is biologically characterized by having at least one or more alpha or pi whorl domains. In a desirable example, this compound is in any of the member of a VIP/GRF peptide family, and this compound is VIP by the most desirable example.

The explanatory view 1 of a drawing is a microphotograph of the multiplex lamellae liposome of this invention.

Drawing 2 shows continuation of the fall in the average arterial blood pressure of the hypertension hamster after processing by bolus injection of VIP of 1.0nmol in SSL.

Drawing 3 shows the effect in the diameter of an arteriole of the suffusion for 7 minutes of 0.1nmol in SSL in a normal-blood-pressure hamster.

Drawing 4 shows the effect of 1.0nmol in SSL poured in for 30 minutes to the nasal cavity of the hypertension hamster.

Drawing 5 indicates an effect VIP in SSL in the neutrophil leucocyte chemotaxis response to formyl-methionyl leucyl phenylalanine (fmlp) peptide, VIP independence, and SSL independent.

The asterisk in; figure showing the fall in average arterial blood pressure using the liposome manufactured by the method of indicating drawing 6 in the Example 3 shows a significant difference statistically between SSL lacking in the inside VIP and VIP of SSL.

DETAILED DESCRIPTION This invention provides the process of the activity biologically liposome products in liposome and an association condition which contain an activity amphiphilic compound biologically. A desirable amphiphilic compound is characterized by having the hydrophilic domain and hydrophobic domain which the hydrophobic domain can meet with a liposome double layer, or were separated to the grade which can meet within a liposome double layer. this invention compound is in this liposome double layer and an association condition, or its thing which are in an association condition within this liposome double layer and which has been biologically acquired for activity conformation is preferred. As for activity conformation, a desired compound tends to affect the normal biological activity through a receptor or ligand recognition, and combination, for example. It is thought that the compound of this invention is characterized by having one or more separate alpha or pi whorl domains which have been divided into the hydrophobic domain and the hydrophilic domain. The desirable compound of this invention is a member of a VIP/GRF peptide family. The most desirable compound of this invention is VIP. the meeting is irreversible although the activity compound is meeting with the liposome double layer biologically -- this compound -- the characteristic of liposome and a compound -- from a meeting with liposome -- promptly -- or

pass time -- it may be emitted.
It lets the film and filter for often obtaining the liposome of desired size pass, and that in which the liposome of this invention has a diameter below 300 nm in advance of contact with an active compound ingredient is obtained compared with the advanced technology including the process of carrying out extrusion molding of the peptide content liposome. The liposome of this size improves liposome, precedes that this incubates with an activity compound biologically, and can be obtained using the extrusion molding step which reduces the size of liposome to a desirable pitch diameter.
The liposome of desired size may be chosen using the art like filtration or other size selecting technics as an exception method. Although the size selection liposome of this invention should have a pitch diameter below about 300 nm, as for them, being chosen is preferred so that it may have a pitch diameter below about 200 nm, and especially the thing for which it has a pitch diameter below about 100 nm is preferred. When liposome products [activity / target / this / biology] are single lamellae liposome, it is preferred to be chosen so that it may have a pitch diameter below about 200 nm. The most desirable single lamellae liposome of this invention has a pitch diameter below about 100 nm. However, the multiplex lamellae liposome of this invention derived from general more small single lamellae liposome is understood to be larger and to be able to have a pitch diameter which is about 1000 nm. The desirable liposome of this invention has a pitch diameter below less than about 800 nm and about 500 nm, and, on the other hand, the most desirable multiplex vesicle liposome of this invention has a pitch diameter below about 300 nm.
The liposome of this invention is publicly known enough, and may be manufactured from the combination of the lipid material containing at least one sort of lipid components which are used for usual by the technical field concerned, and which carried out the covalent bond to water-soluble polymer. As lipid, the comparatively hard kind like sphingomyelin or the fluid kind like the phospholipid which has an unsaturation acyl chain is mentioned. Polymer of this invention SSL art and polyvinyl alcohol, Poly lactin acid, polyglycolic acid, a polyvinyl pyrrolidone, polyacrylic amine, It is publicly known at the technical field concerned about art useful to ** which increases the circulation half-life of protein including the quality of synthetic tallow which has polyglycerol, poly AKISOZURIN, or a polymer head group, and which compound used for usual may also be included. The most desirable polymer of this invention is PEG which has a molecular weight between 1000 thru/or 5000.
As desirable lipid for manufacturing liposome by this invention, A covalent bond is carried out to a PEG (PEG-DSPE) phosphatidyl chlorin (PC) and phosphatidylglycerol (PG), and the JISUTE roil phosphatidylethanolamine which uses cholesterol (Chol) together further is mentioned. As for the lipid for manufacturing the liposome of this invention, and the combination of cholesterol, according to the desirable example of this invention, the mole ratio

of PEG-DSPE:PC:PG:Chol comprises 0.5:5:1:3.5.

Stability and biological activity are improved and the liposome manufactured by the method of this invention is characterized according to a useful thing for various therapies. According to one example, this invention includes the constituent in which an activity amphiphilic compound has anti-oxidant activity, antiaging, anti-wrinkles formation, or wound healing ability biologically and for which activity liposome products are contained biologically. This kind of constituent can have cosmetics or the medicine characteristic. Activity VIP is biologically contained in a desirable cosmetic composition. This invention also provides the oral controlled release pharmaceutical preparation for the therapy of the therapy of a gastrointestinal disease in which the process includes the process of enclosing activity liposome products with this biology target, during further enteric covering. This oral controlled release pharmaceutical preparation is useful to various gastrointestinal diseases containing what chosen from the group which consists of an inflammatory internal disease, the chronic constipation, a Hirschsprung's disease, the achalasia, suckling hypertrophic pyloric stenosis, and an ulcer. Activity VIP is biologically contained in the desirable oral pharmaceutical preparation. The liposome preparation which contains activity VIP biologically is also a promising remedy for the condition like asthma, systemic hypertension and the pulmonary hypertension, the scleroderma, myocardial ischemia, the impotentia, and baldness. Furthermore, this invention provides the method of saving the body organ, organization, or cell strain for storage and transplantation in a recipient including the process of incubating the organ in the liposome composition containing VIP.

Furthermore, this invention manufactures the activity biologically liposome products in liposome and an association condition which contain an activity amphiphilic compound biologically by the method of this invention, Subsequently, the method of medicating a target tissue with an activity amphiphilic compound biologically characterized by the process of medicating a target tissue with these an effective dose of liposome products remedially is provided. The liposome products of this invention The inside of a vein and an artery, or the aerosolization, What is necessary is just to prescribe a medicine for the patient directly in endotracheal and a joint, in a nasal cavity, by the local administration to the membrane like the tunica mucosa oris, gastroenteric lower membrane, and a conjunctiva, and administration to a target at the time [according to / spraying, inhalation, or aeration], although it is not taking orally, transderma, hypodermic, and a thing limited.

When especially activity compounds have especially short half-life biologically while the compound circulates, or biological activity falls in a cure, compared with administration of an independent compound, a medicine can be prescribed for the patient with the dosage level reduced notably. For example, in VIP in SSL and an association condition, it is expectable that biological activity enhancement and extension are shown compared with VIP independent

administration. Generally, an effective dose of VIPs are [50 thru/or 75 % of the weight of abbreviation] biologically [in SSL] lower than the biological effective dose of VIP in the inside of solution. Although the biological activity compound is meeting with SSL, in order to determine a biological effective dose required to attain the equivalent result given with the compound prescribed for the patient by a conventional method, this liposome product must be examined. Usually, probably, it turns out that the biological effective dose of a certain compound is helpful as the starting point in an effective dose of determination of the compound in SSL, when sending a person skilled in the art with a conventional method. Therefore, in order to determine the minimum dose required to attain desired biological effectiveness, what its equivalent and dose lower than it in SSL will be sufficiently effective only for being only in accordance with the custom will be guessed strongly. Probably, VIP in SSL for attaining an equivalent effect is 5 thru/or 10 mg, if the conventional administration requires the dose which is 20 mg, for example in VIP administration. Typically, probably, VIP's biological effective doses by which vein administration is carried out are a total amount per day of 0.01 thru/or 50 mg or 0.1 thru/or 500mgVIP with a capsule gestalt.

by meeting with activity SSL of a compound, it is expected biologically [this invention] about 50% thru/or that it will exceed 100% and the size of the biological effectiveness of this compound will increase of the effect observed following administration compound independent [this]. Similarly, in a meeting with SSL of this invention, making biological effectiveness maintain for a long time is expected.

The improved constituent for diagnosis in which this invention furthermore contains multiplex vesicularity biological activity liposome products, And the activity biologically liposome products in the multiplex lamellae liposome manufactured by the method of this invention and an association condition which contain an activity amphiphilic compound biologically are manufactured, A target tissue is medicated with these liposome products of a diagnosis top effective dose, and those directions characterized by the process of subsequently detecting the incorporation or interaction of liposome in this target cell are provided. According to one mode of this invention, this target cell is a tumor. In one mode of this method, the sign of this liposome product is carried out so that the sign chosen from the group containing the compound which raises a radioactive label, fluorescent labeling, un-labeling fluorescently, a color, or magnetic resonance imaging (MRI) can detect. According to the desirable example of this invention, this liposome product is detected by sound wave reflection. Although the liposome products for diagnosis for detection by sound wave imaging generally have a pitch diameter below about 1000 nm, this liposome product for diagnosis has a pitch diameter below 600 nm, and has a pitch diameter below about 300 nm most preferably.

This invention Inflammation, hypertension, allergy, an Alzheimer disease, Atherosclerosis, a disease with built-in inflammatoriness, the chronic constipation, a Hirschsprung's disease,

Promotion or control of the therapy of the achalasia, suckling hypertrophic pyloric stenosis, and an ulcer, and cell growth, It is manufactured by the method of this invention for inhibition of promotion of inhibition of apoptosis, a body organ, or the wound healing of an organization, an organ, or tissue rejection, and use of the activity biologically liposome products which contain an activity amphiphilic compound biologically is provided.

To the provisional application 60th by which it is considered that the indication is some of these specifications / No. 014 or 363, the result of use of the VIP meeting liposome by this invention is indicated. Especially VIP-PEG-liposome was manufactured as following. DSPE (molecular weight 1,900), PG, PC, and cholesterol (mole ratio 0.5:1:5:3.5) which were combined with PEG were dissolved in chloroform using the round bottom flask. This solution was dried in the rotation evaporator overnight, and the obtained film was dried overnight. This lipid membrane was rehydrated stirring using pH six to 7 physiological saline, and, subsequently the sonication was carried out for at least 5 minutes. The aperture extruded the liposome preparation formed in this way through the accumulated Nucleopore filter which are 200mn, 100 nm, and 50 nm, respectively until the average size of the PEG-liposome measured by quasi-elastic light scattering was set to 80 to 100 nm. This mixture was quick-frozen for at least 20 minutes in ethanol or an acetone dry ice bath, and, subsequently it was made to freeze-dry under the same conditions overnight in addition to the liposome preparation which had the trehalose which are VIP and a chill protecting agent extruded in a polypropylene tube. Isolation VIP was separated from VIP-PEG-liposome using A-5 m of Biogel column chromatography. The size of the PEG-liposome in the original solution and VIP-PEG-liposome was measured by quasi-elastic light scattering. The lipid concentration of the PEG-liposome in the original solution and VIP-PEG liposome was measured with inorganic-phosphoric-acid assay. The VIP concentration in VIP-PEG-liposome was measured with ELISA assay.

1% sodium dodecyl sulfate which is a surface-active agent was added to some VIP-PEG-liposome preparation in order to measure the VIP concentration in VIP-PEG-liposome, and to separate VIP who is meeting before a fixed quantity. PEG-liposome and a 1% sodium-dodecyl-sulfate independent did not block ELISA assay. From the example to which the preliminary test using these pharmaceutical preparation is not restricted, the increase and extension of biological effect to the target tissue of the mammals like a postscript were shown.

Furthermore, in this provisional application, bolus intravenous injection of the VIP-PEG-liposome compound of 1.0nmol reduced the average arterial blood pressure (MAP) of the spontaneous hypertension hamster. ; drawing 2 A this result is indicated to be to drawing 2 A and 2B in this specification shows the fall of actual arterial blood pressure, and drawing 2 B shows % change. ; asterisk whose data is average value ** standard deviation shows the significant value statistically [when 5% or less of percentage of risk compares with contrast]. a result is significant -- reduction of a target and continuous average arterial blood pressure was

shown gradually, the minimum score was reached within 2 hours after VIP-PEG-liposome pouring, and it continued during the observation period of 7 hours.

According to another experiment, when the suffusion of the cheek pouch of a normal-blood-pressure hamster was carried out for 7 minutes with the VIP-PEG-liposome composition of 0.1nmol, in in situ, the significant increase in an average diameter of the artery was caused. The result of this experiment is shown to drawing 3 by data, and the significance of results is shown in said drawing 2 A and 2B. The significant increase from the fiducial value of the diameter of an arteriole was accepted, and optimum was observed in less than [suffusion start 5 minute]. The diameter of the artery returned to the fiducial value 9 minutes after the **** stop.

Furthermore, in another experiment, when the VIP-PEG-liposome composition of 1.0nmol was injected into the nostril of a hypertension hamster for 30 minutes, the fall of arterial blood pressure took place and it was maintained for at least 150 minutes. These results are shown in drawing 4. The fall to a target and the continuous normal range of average arterial blood pressure was accepted gradually, and it was maintained during the observation period over 2.5 hours.

At the last, the influence of the VIP-PEG-liposome to neutrophil leucocyte chemotaxis was considered using 2 tub type device usually used for the chemotaxis analysis in in vitro as another experiment. The result of the experiment is shown in drawing 5. Movement of the neutrophil leucocyte from an upper tub to the lower tub reacted to formyl-methionyl leucyl phenylalanyl (fmlp) peptide of the lower tub was carried out as first base contrast value. There is almost no neutrophil leucocyte movement to the medium (the balanced salt solution of a hank, HBSS) and VIP itself of a lower tub, and very slight neutrophil leucocyte movement was accepted to the VIP-PEG-liposome and PEG-liposome of a lower tub. When both neutrophil leucocyte and VIP were added to an upper tub, significant movement was accepted to fmlp of a lower tub, but as compared with the case where both neutrophil leucocyte and PEG-liposome are added to an upper tub, it was the cell migration of the slightly low level. Finally, when VIP-PEG-liposome was added to an upper tub with a cell, neutrophil leucocyte movement to fmlp decreased even on the level which can almost be disregarded. These results show that VIP-PEG-liposome can check the chemotaxis of fmlp reactivity neutrophil leucocyte movement.

This invention is further clarified by the following examples. When a spontaneous hypertension hamster is medicated with Example 1, the incorporation to the activity liposome of VIP peptide is a comparison example which indicates the state of the art concerned of explaining increasing the period and scale of peptide activity biologically. Example 2 is an equivalent thing which is in the liposome (SSL) stabilized in three dimensions and an association condition by the method of this invention concerning an experiment of activity peptide biologically.

This liposome brings about a much more dramatic increase in peptide activity.
In Example 3, the exception method for preparation of SSL is provided by this invention it is indicated to be to produce the peptide activity of the level with which different preparation art differs dramatically. In Example 4, the analysis method of the morphological characteristic of the liposome manufactured by the method indicated in Example 3 is provided. Example 5 is related with the strange method for manufacturing SSL which has the peptide which has biological activity that the simplification of a manufacturing process does not influence the activity of peptide in in vivo at all. Example 6 has indicated use of the product for the use in sound wave reflective imaging based on the echo reflection property of liposome, and the liposome products for diagnosis.
Biological activity of peptide in the liposome of the example 1 former (comparison example)
The method of the advanced technology for the incorporation to VIP's liposome was reproduced for the purpose of providing the foundation for comparison with the method of this invention by this example. In old research, we decided [in / since bearing a certain role was suggested when VIP controlled stress of the vasomotion / in situ peripheral thin circulation] to examine first the VIP activity as a function of the carrier used in order to dissolve and send peptide. By enclosing VIP with whether the vasodilatation in peripheral thin circulation of a spontaneous hypertension hamster is caused further especially by VIP's local administration, and conventional single lamellae liposome, The first experiment was conducted in order to measure whether it can adjust any of the accepted reaction they are.
The normal-blood-pressure control group (n= 20) which the spontaneous hypertension hamster (n= 21) and the age, and the genetic factor of the male which matured suited was purchased from Canadian Hybrid Farms, Halls Harbor, NS, and Canada. As preparation, pentobarbital sodium (6 mg/100g weight) was injected intraperitoneally, and it applied to the animal at anesthesia, and tracheostomy was given in order to make spontaneous breathing easy. For one by one pouring to anesthesia (2 thru/or 4 mg/100g weight / time), Kanew ration was carried out to the left thigh vein. The catheter was inserted in the left femoral artery in order to record systemic arterial blood pressure and a heart rate. Body temperature was supervised so that 37 thru/or 38 ** might always be maintained [be / it / under / experimental period / letting it pass] using a heating pad.
In order to visualize thin circulation of a cheek pouch, [Gao et al. and Life for which the method indicated to old was used. Sci.64 :P L274-PL252. (1994); Mayhan. And Joyner, Microvasc.Res.28:159-179(1984);Mayhan and Rubinstein, Biochem.Biophys.Res.Commun.184:1372-1377(1992);Raud, and Acan. Physiol.Scand.Suppl.578:1-58. (1989); Rubinstein. Am.J.Physiol.261(Heart Circ.Physiol.30):111913-111918(1991); and Mayhan and J.Lab.Clin.Med.125:313-318(1995); -- Rubinstein et al.. And Suzuki et al., Life Sci.57:1451-1457(1995)]. Expedient, the left cheek

bag was opened on the base plate of a small plastic, and in order to expose a cheek pouch film, the outside skin was cut open. The connective tissue layer of the non-blood vessel was removed, the box of the plastic was placed on the base plate, and it fixed by suturing the skin around an upper tub. The cheek pouch film exposed between :base plate in which the complex which consists of three layers is formed by this preparation, an upper tub, and two plates. It is connected to the storage kettle into which the warmed 2 carbonate buffer solution (37 to 38 **) was put, and the suffusion of a continuous cheek pouch is possible for an upper tub. Buffer solution was continuously aerated with nitrogen and 5% carbon dioxide (pH 7.4) 95%. It is connected to a constant infusion pump (Sage Instruments, Cambridge, MA) through a cross valve, and the controlled medication is possible for a tub again. In next research, this animal preparation method can be similarly used, as shown below.

The liposome containing VIP, Gao et al. and Life Sci.64 :P L274-PL252(1994);Gregoriadis and Florence, and Drugs 45:15-28(1993); -- MacDonald et al. and Biochem -- Biophys.Acta. 1061: It prepared by the method of 297-303(1991); and Suzuki et al., and Life Sci.57:1451-1457 (1995). Expedient Egg yolk phosphatidylcholine (Sigma, St.Louis, MO), Egg yolk phosphatidylglycerol (Sigma) and cholesterol (Sigma) mix the lipid composition thing (the total lipid content of 5 mg) which comprises the mole ratio of 4:1:5 in chloroform (Sigma), and the solvent was evaporated and was dried. The dried lipid membrane was re-suspended using a vortex mixer and the sonication in the 0.15-mol sodium chloride solution of 100microl containing 0.7-mg VIP. A suspension solution is covered over the freezing-dissolution of five cycles using a dry ice ethanol bath, It extruded 9 times through the polycarbonate filter (aperture 3 micrometer;Nuclepore, Pleasanton, CA) of two sheets using the LiposoFast device (syringe capacity 0.5 ml;Avestin, Ottawa, ON, Canada).

Liposome uses a disposable gel filtration column (Econo-pac 10DG, polyacrylamide gel, 10 ml of bed volume), [MacDonald et al., Biochim.Biophys, Acta 1061:297-303] which were collected with 0.15N sodium chloride (1991); liposome fractionation was collected in void volume and saved at 4 ** to use.

As follows, change of the diameter of the artery was measured. Upper irradiation of the microcirculation in bursa buccalis was carried out using the optical fiber light source, and it was observed under the microscope of NIKON (Nikon). Under the microscope, the picture was projected into closed circuit television systems provided with a low illumination television camera, a television monitor, and a videotape recorder (Japan, Yokohama, Panasonic). The diameter of an inner surface of the secondary arteriole in bursa buccalis is a video micrometer (measured from the video presentation of the shape of microscopic features using VIA-100 and a BEKKERA instrument (Boeckeler Instruments, a taxon, Arizona).). Proofreading of the magnification of a video system uses a microscope stage micrometer, in order to obtain a detailed vessel diameter by the micrometer, and it is line cotton. The container was chosen for

the clear observation on a monitor screen, and the position in the arteriole branch pattern in bursa buccalis. In each animal, change of the internal-surface caliber was measured using the arteriole portion same during an experiment. In a certain research, once measurement of the diameter of an arteriole returned from former intervention to the baseline, the animal was used for two or more processing groups.

VIP is in the state which carried out cyst by liposome independently, and it is 0.05 mol or 0. By the time it back-overflowed and applied peptide for 7 minutes by the VIP concentration of 1-mol peptide, 30 minutes or more passed. It opted for change of the midst and the subsequent diameter of an arteriole as mentioned above before VIP's partial application. Life Sci.64 besides [Gao (Gao) which was a thing based on former research in VIP's concentration used for these experiments :P Life Sci.57:1451-1457(1995)] besides L274-PL252(1994);Suzuki.

In the result, overflowing of VIP itself in both concentration showed that the maximum response was observed within 4 minutes after the start of overflowing in relation to the effective vasodilatation of the hamster of normal blood pressure. After VIP's overflowing stopped, the diameter of an arteriole returned to the baseline within 1 minute. By contrast, VIP itself's overflowing did not express an effect with the diameter of an arteriole of the hamster of natural hypertension. This blunt response to VIP in a hypertension animal is not attributed to the un-specific damage over an inner bark. The inner-bark independent vasodepressor [maven [in / in the reason / nitroglycerine and bursa buccalis] (Mayban) and the Rubinstein (Rubinstein) name, Biochem.

Biophys.Res.Commun.184:1372-1377 (1992); Rubinstein (Rubinstein)

It is because others and Am.J.Physiol.261(Heart Circ.Physiol.30):111913-111918(1991)] causes the same blood pressure fall in both groups.

Although it was the same quantity, by VIP's overflowing by which cyst was carried out to liposome, the positive pressure nature animal showed an effective concentration dependence stratified operation and extension of the blood pressure fall effect as compared with VIP itself. After it was detected in 4 minutes from 3 minutes after an overflowing start and overflowing stopped, effective vasodilatation maintained the maximum response for about 9 minutes. In the hamster of natural hypertension, VIP by whom liposome cyst was done stored the same effective blood pressure fall effect as what was observed for the positive pressure nature animal. After it was detected within 4 minutes after the overflowing start and overflowing stopped, the effective blood pressure fall maintained optimum 3 minutes or more. Though VIP's liposome cyst could recover the blood pressure fall effect of peptide of the hamster of natural hypertension like what is observed for a positive pressure nature animal, the duration of the effect was short much.

By these results, the vasodilatation which appears by VIP in the circumference microcirculation of a positive pressure nature hamster changes from regulation of the duration to regulation of

two elements, i.e., the size of the response to the 1st, and the 2nd. Although the former is expressed in aquosity and a lipid environment, Only when VIP is divided into a lipid bilayer, it is observed by the latter and [Gao (Gao) etc. Life Sci.64 :P L274-PL252 (1994); Gregory Addis (Gregoriadis) and Florence (Florence), Drugs 45:15-28 (1993); McDonald's (MacDonald) etc. Biochim.Biophys.Acta 1061:297-303 (1991); Musso (Musso) etc. Biochemistry 27:8174-8181 (1998); Noda (Noda) etc. Biochim.Biophys.Acta1191:324-330 (1994); Robinson (Robinson) etc. Biopolymers 21:1217-1228 (1982); Solo BIEFU (Soloviev) etc. J. Hypertens.11.623-627 (1993); Life Sce.57:1451-1457(1995)] and this besides Suzuki (Suzuki) can also provide the environment of being suitable for pi spiral formation in a VIP molecule. [Noda (Noda) etc. Biochim.Biophys.Acta 1191:324-330 (1994); Biopolymers 21:1217-1228(1982)] besides ROBINZON (Robinson). It turned out that the lipid dependence ingredient of the VIP induction vasodilatation in circumference microcirculation is not looked at by the hamster of idiopathic hypertension for the reason which is not completely clear.

Example 2 The feature of the opposition-pairs thing operation in liposome stable in three dimensions Although the VIP cyst in conventional liposome recovered the capacity of peptide and explained having induced the vasodilatation of the hamster of natural hypertension, Change of Sai's VIP activity relevant to the liposome where this invention was stabilized in three dimensions was examined.

As Example 1 described intrinsically, the positive pressure nature animal was prepared with the following change. Apply anesthesia to the male (n= 28; 120 to 140 g weight) of the adult of a golden hamster with pentobarbital sodium (6 mg / weight of 100 g, i.p.), and cannula is inserted in a femoral vein, The marker in a vessel, dextran (FITC-dextran is dissolved in 1.0 ml of salt water; molecular mass 70kDa; 40mg / weight of 100 g, 1-minute or more administration) of a fluorescein isothiocyanate sign, and supplemental anesthesia (2-4mg / weight of 100g/hour) were prescribed for the patient. The procedure which stated the change in the microcirculation of bursa buccalis in above-mentioned Example 1 in order to make it visible was adopted.

RIPO z-MU (SL) stable in three dimensions was prepared as follows. Egg yolk phosphatidylcholine (sigma), egg yolk phosphatidylglycerol (sigma), cholesterol (sigma), and JISUTE aroyl. phosphatidylethanolamine (mole-ratio 5:1:3.5:0.5; -- a phospholipid content.) The polyethylene glycol (molecular mass 1900) combined with 17 mol is dissolved under chloroform, Life Sci.64 besides mixed [Gao (Gao) :P L274-PL252 (1994); [RAJIKKU (Lasic) and Martin (Martin),] Am.J.Physiolo[besides Srealth Liposomes, CRC Press.Inc.;Boca Ratoron.Florida, and 1995. Suzuki (Suzuki)].271:H282-H287(1996)]. The solution was evaporated in the vacua in the temperature of 45 ** in the rotation evaporator overnight. With 250 ml of salt water, the lipid membrane obtained as a result is rehydrated, and eddies, Organ bath ultrasonication was carried out for 5 minutes, and it extruded via the polycarbonate filter

accumulated using the RIPOZO first device (continuation caliber : 50 nm: [200nm, 100 nm, and] ABESUCHIN (AVESTIN) and ink, Ottawa, Ontario, Canada). Human being's VIP (0.4 mg) and trehalose (30 mg), and an antifreeze are extruded, and it adds to suspension. then -- freezing in an acetone dry ice organ bath -46 -- under the fixed pressure, it was lyophilicity-ized at the temperature of -46 ** overnight (the FO scene (Foreseen) 6, love KONKO, Kansas City, Missouri).
Then, the "cake" lyophilicity-ized was suspended by 250 ml of deionized water. SSL-related VIP is separated from isolation VIP by column chromatography (A-5 m of Biogel (Bio-Gel)). It was stored for a maximum of 15 days at Bio-Rad Laboratories (Bio-RadLaboratories), Richmond, California, and the temperature of 4 **. the size of SSL was 250**50 nm, as determined by quasi-elastic light scattering (a Nicomp model 270 submicron particle sizer.) Phospholipid in Pacific scientific (Pacific Scientific), Menlo Park, and California SSL, [Cates em determined by barrulet inorganic-phosphoric-acid salt inspection (Kates.M.), Theque NIKUSU yne lipid logy (Techniques in Lipidology), work and work (Work and Work) (Bds.), Els Baeyer (Elsevier)
: New York, New York (1972), 354 pages, or 356page]. After the VIP concentration in SSL dissolved SSL with 1% of sodium dodecyl sulfate, it was determined by the available ELISA inspection kit on available commerce on commerce. A recovery rate is 50% to phospholipid 30% to VIP.
The 0.007VIP mole ratio of phospholipid was obtained.

A decision of the diameter of an arteriole was made as the above-mentioned Example 1 described. In the animal of the 1st group, 0.42-mol VIP and 0.85-mol VIP in SSL overflowed in arbitrary order for 1 hour. Life Sci.57:1451-1457 (1995) besides [which passed for at least 45 minutes until it overflowed VIP within SSL after that] [Suzuki (Suzuki); Am.J besides Suzuki (Suzuki).
Physiol.271: H282-H287(1996)]. The diameter of an arteriole was measured at intervals of 5 minutes for every minute, when overflowing VIP within SSL just before overflowing. It was shown by former observation that overflowing salt water independent [in the whole period of an experiment] is seldom related to change of the diameter of an arteriole. In the animal of another group, SSL of the empty of concentration equivalent to VIP in SSL (0.1 mol) or 0.1-mol VIP in SSL (18-mol [/ml] phospholipid) was overflowed for 7 minutes.
By overflowing in SSL the animal of the 1st group that has 0.42 mol and 0.85-mol VIP over 1 hour, the increase in concentration dependence extension with a remarkable diameter of an arteriole was performed. The diameter of an arteriole returned to the baseline level in 50 minutes, after overflowing of VIP in SSL stopped. Overflowing of 1 hour using empty SSL seldom stored the effect in the diameter of an arteriole.

By overflowing the positive pressure nature animal of the 2nd group using 0.1-mol VIP in SSL, it was observed in the 1st group and the diameter of an arteriole also increased reliance from the baseline considerably to the low grade. The diameter of an arteriole returned to the baseline in 13 minutes, after overflowing of VIP in SSL stopped. Overflowing of empty SSL seldom stored the effect in the diameter of an arteriole.

Even when the vasodilatation of 1 hour was larger than what was observed by overflowing for 7 minutes, the result of changing far exceeding a baseline was shown by by using 0.1 mol of peptide.

The vasodilatation effect of VIP in SSL arises selectively according to the un-specific damage over a detailed blood vessel, In order to determine whether a micro molecule flows out of bursa buccalis as a result [Gao (Gao) etc. Life Sci.54 :P L247-PL252 (1994);. Loud (Raudo) Using Acta.Physiol.Scand.Suppl.578:1-58(1989)] and two indices, as mentioned above under control and an experimental condition, Life Sci.54 besides [Gao (Gao) which determined to sweep away polymers from bursa buccalis :P L247-PL252 (1994); loud (Raudo), Acta, Physiol.Scand.Suppl.578:1-58(1989)]. It was the determination of the fluorescence around the venule of a back capillary tube "point", or the number of disclosure parts the 1st, and was the determination of the FITC dextran eradication from the 2nd bursa buccalis.

After overflowing an animal in the balanced period for 30 minutes using bicarbonate buffer solution, FITC dextran was prescribed for the patient into the vein. VIP in SSL (0.1 mol) overflowed for 7 minutes after that, and the disclosure part was determined every 7 minutes at first, and was determined at intervals of 5 minutes in 60 minutes after that. an eradication of FITC dextran -- before overflowing of VIP in SSL, and under overflowing -- every 5 minutes -- [determined after overflowing for 60 minutes -- Life Sci.54 besides Gao (Gao) :P L247-PL252 (1994)].

The result that overflowing of mol VIP in SSL did not relate to the disclosure part formation which is a foregone conclusion was shown. Similarly, the eradication of FITC dextran under overflowing of salt water was intrinsically [as the eradication under overflowing of VIP in SSL] the same.

The extended remarkable concentration dependence vasodilatation was shown with the combination of these results by VIP overflowing in SSL on the bursa buccalis of a hamster. This response did not relate to un-specific damage over detailed vascular endothelium. It was because the diameter of an arteriole will return to a baseline and VIP in SSL will not cause the polymers outflow from the post capillary venule in bursa buccalis, if VIP overflowing in SSL stops. In the illness to which VIP in SSL spoils inner-bark dependence vasodilatation, such as hypertension, congestive heart failure, diabetes mellitus, and impotence, by these results, The [pole (Paul) and Ebadi (Ebadi) to whom it was shown that it was usable when recovering the vessel reactivity in circumference microcirculation, Neurochem.Int.23:197-214 (1993);

Am.J.Physiol[besides Suzuki (Suzuki)].271:H282-H287(1996)].

Example 3 Although it told VIP in comparison SSL of the opposition-pairs thing operation as a liposome process function that the opposition-pairs thing operation improved in the VIP process in conventional liposome, The optimal ingredient determined the process, and since an opposition-pairs thing operation of VIP in SSL was characterized further, it examined another process.

Two different liposome processes were used. in both processes -- lipid JISUTE aroyl phosphatidylethanolamine (PEG-DSPE) (SEKUASU Pharma SUCHI callus (Sequus Pharmaceuticals). Menlo Park.) California and egg yolk phosphatidylcholine (PG) (a sigma chemical company (Sigma Chemical Co.).) St. Louis, Missouri, and egg yolk phosphatidylglycerol (PG) (a sigma chemical company (SigmaChemical Co.).) St. Louis and Missouri were combined with cholesterol (Sigma Chemical Co.), St. Louis, and Missouri in the PEGDSPE:PC:PG:Chol mole ratio 0.5:5:1:3.5.

The mixture was mixed to the chloroform in a round bottom flask, the solvent was evaporated within the rotation evaporator (love KONKO (Labconco), Kansas City, Missouri) at the temperature of 45 **, and complete drying of the mixture was carried out by the vacua overnight.

In the 1st process (this is not planned by this invention), the beginning was mixed with the lipid component, and VIP was extruded after that, repeated refrigeration and an answer, and generated liposome. In short, the dry lipid film was rehydrated with 250microl0.15M salt water (0.9%w/wNaCl) containing 0.4mgVIP (an American PEPICHIDO company, Sunnyvale, California). The mixture eddied and performed refrigeration and an answer 5 times in the 172.5W water bath ultrasonic treatment apparatus (Fischer SAIENTE Fick, ITASUKA, Illinois) and the acetone dry ice organ bath. Suspension was extruded via the polycarbonate filter using the RIPOZO first device (200 nm in an aperture, ABESUCHIN (AVESTIN) and Inta, Ottawa, Ontario, Canada). About liposome company VIP, it is column chromatography (BioGel A-5m, Bio-Rad Laboratories (Bio-Rad Laboratories)).

It stored at 4 ** until it dissociated from isolation VIP and used it by Richmond and California. Column elution was performed using the above-mentioned 15M 270 salt-water solution. A Nicomp par chicle sizer (par chicle sizing systems (Particle Sizing Systems) and Santa Barbara California) is used for vesicle size, It turned out that it is determined by quasi-elastic light scattering [J.Pharm.Sci.In press (1996) besides pore RUKAN**ONYUKUSEI (Alkan-Onyuksei)], and the liposome generated by this method has an average diameter of 224**36 nm.

In the 2nd process planned by this invention, the lipid mixture was extruded first and mixed after that the liposome and VIP who were formed. The dry lipid membrane generated as mentioned above in short was rehydrated with 250-ml0.15M salt water by the state where

there is no VIP. The mixture eddied, was ultrasonicated in the organ bath for 5 minutes, was extruded via the laminated poly car PONETO filter whose apertures are 200 nm, 100 nm, and 50 nm, and obtained the BESHITARU size of about 80 nm. VIP (0.4 mg) and the trehalose (30 mg) (the sigma KEMIKAU corporation (Sigma Chemical Co., St. Louis, Missouri) was added in powder shape, and extrusion suspension was formed.) as an antifreeze The mixture was cultivated at 2 hours or 4 ** with the room temperature overnight, was frozen in the acetone dry ice tub, and was about freeze-dried at -46 ** with the pressure of $5x10^{-3}$MBar overnight (love KONKO (Labconco "Freezone 6", Kansas, Missouri).). Freeze-dried "cake" was re-suspended by 250-micrometer deionized water.

During freeze-drying, VIP and phospholipid bilayer were close and promoted passive drugs increase in quantity.Column separation and a storage condition were the same as the above. It turned out that the liposome generated by this method has an average diameter of 250**50 nm with a described method. This has suggested that freeze-drying enables vesicle fusion. The VIP concentration of liposome is a VIPELISA inspection kit (and). [Peninsula Laboratories] It is determined by Belmont and California after the processing using 1% of sodium dodecyl sulfate, and phospholipid concentration, Verrett (Barlett) inorganic-phosphoric-acid salt kit [em Cates (M-Kates) Techniques in Lipidology, a work and work (Eds)

From 354 pages to Els Baeyer (Elsevier), New York (1972), and 356page] As for about 30% of start VIP, both were understood that liposome company is carried out to the generating method, about 50% of start phospholipid was recovered, it was collected and about 0.004-mol VIP / mole ratio of phospholipid were given.

The in vivo experiment of two form was conducted and VIP's blood pressure fall and hypertension effect in the liposome generated by two methods were determined. In the 1st continuation experiment, VIP's opposition-pairs thing operation in a liposome process was examined as vasodilatation, and VIP's effect in two liposome processes in mean arterial pressure was measured in the 2nd continuation experiment.

In the 1st experiment, VIP's opposition-pairs thing operation in a liposome process was measured as an operation of change of the diameter of an arteriole in the bursa buccalis of a hamster. the golden hamster (SASUKO (Sasco) and Omaha.) of a grown-up male Nebraska is generated as mentioned above and [Suzuki (Suzuki) etc. Life Sci.57 (15):1451-1457 (1995); Suzuki (Suzuki) etc. Am.J.Physiol.271:11282-H287 (1996); Suzuki (Suzuki) etc. Anesthesia was applied via the femoral vein which inserted Am.J.Physiol.In press(1996)] and cannula with pentobarbital sodium (2-4mg / weight of 100g). inserting cannula in a femoral artery -- a transducer and a strip chart recorder (the model 260, good instrument systems ink (Gould Instrument Systems Inc..)) Whole body system arterial pressure and a heart rate were recorded using a BARI view and Ohio. Life Sci.57 (15) besides [Suzuki (Suzuki) where the microcirculation of the bursa buccalis of the animal model established in order to investigate

the blood vessel operation effect of the neuropeptide of natural grade was visualized as mentioned above
: 1451-1457 (1995); Am.J.Physiol.In press(1996)] besides Am.J.Physiol.271:11282-H287 (1996); Suzuki (Suzuki) besides Suzuki (Suzuki). The diameter of a wall of the secondary arteriole in the bursa buccalis of a hamster was measured from the video display of the shape of microscopic features using a video micrometer (VIA100; BEKKERA INSU vine face (Boeckeler INstruments), a taxon, Arizona). In each animal, the diameter change was measured using the artery portion same during an experiment. First, the bursa buccalis of the hamster used the carbonate-bicarbonate buffer for the balanced period for 30 minutes, and overflowed for 7 minutes after that, using each of above-mentioned liposome pharmaceutical preparation 0.4 ml.
Although VIP of the liposome generated by the 1st method was outside the range of this invention, the increase in a considerably different diameter of an arteriole from 0.1 mol dissolved in salt water, i.e., the observation reported a priori using about 10% of VIP, was not brought about. Although it is the same method, this observation, As compared with the observation before VIP in conventional liposome was generated in the state which showed the prolong effect whose how to twist an extrusion process improved at natural grade, [Suzuki (Suzuki) etc. Since the loss of VIP's activity in SSL generated by Life Sci.57 (15):1451-1457 (1995)] and this invention is permitted, three possibilities are suggested.
That is, they are an extrusion process, a lipid presentation, or smaller vesicle size. Unless SSL generated by this method improved in the diameter of an arteriole or brings about the extended effect, it is not concerned with a reason, but this result becomes important when it is shown that SSL generally does not follow this invention.
By the 2nd method, VIP (0.1 mol) in the liposome moreover generated within the limits of this invention meant that the diameter of an arteriole increased from the base line value considerably, and this increase was continued for 9 minutes to 16 minutes, after overflowing stopped. This result was closer to the observation before using conventional liposome [Life Sci.57 (15):1451-1457 (1995) besides Suzuki (Suzuki)].
The following procedures were followed when examining VIP's duration and effect in two liposome pharmaceutical preparation in mean arterial pressure. From the Canadian hybrid firm (a hole harbor, Nova Scotia, Canada), the hamster of the male of the adult of natural hypertension (n= 12) came to hand. Each carried out pouring administration of three test pharmaceutical preparation which is about 500microl, the liposome generated by the 2nd method of the above, and the liposome without VIP and VIP in solution over 1 minutes or more at the femoral vein. Continuation anesthesia of an animal restricted the experimental period by 6 hours.
After pouring in 0.1-mol liposome company VIP, it was observed in 2.5 hours of the beginning

that average arteriole pressure decreases to 50% remarkably on a target gradually, and as shown in drawing 6, it continued this state at the experiment observation period of 6 hours. When VIP in solution or empty liposome was used, the remarkable effect of average venous pressure was not observed. With these data, it was suggested that VIP in SSL prescribed for the patient into the vein normalized the mean arterial pressure of the hamster of natural hypertension well for at least 6 hours. A dose required to produce normal blood pressure in an interesting thing, Although VIP of the same quantity [in / as compared with former observation, it is dramatically low, and / conventional liposome] dropped the mean arterial pressure of the positive pressure nature hamster 30%, [Gao (Gao) etc. Life Sci.54 :P L247-PL252(1994)] may attribute this observation to the higher sensitivity of the hamster of natural hypertension to VIP.

Since SSL of the same presentation generated by the method (namely, the 2nd method) of this invention and size held VIP activity, to the loss of an opposition-pairs thing operation, it was suggested to the 1st liposome generation by this result that extrusion is the cause. The experiment proof before Inta Lukin 2 was shown as what loses not less than 25% of activity after extrusion as for this possibility is J Immunother besides the [keddah (Kedar), although it was in agreement. J Pharm Res.9(2):260-265 (1992) besides [UDORU (Woodle) which was contradictory to observation that vasopressin is seldom influenced by 16:47-59(1994)] and extrusion
].
For the morphometric assessment of ** prepared by the method of both which were stated to the morphometric assessment example 3 of example 4 SSL, Liposome was prepared for the ****** fracture to the standard practice already reported in J firm SCI (J. Pharm.Sci.), such as alkane ONYUTASERU (Alkan-Onyuksel) of issue, in 1996. It was temporarily frozen in the liquefaction nitrogen in which the liposomal suspension drop was cooled Freon 22, and was fractured using a -115 ** balzers (Balzers)BAF301 Friesen etching unit, and was coated with platinum and carbon. It was washed by the minimum of two modification of a sodium hypochlorite, and washed and dried with distilled water, and replicase was collected and examined with the copper lattice of 200 meshes, and was photoed at 80 kilovolts with the JEOL100CX transmission electron microscope.
The test result of SSL adjusted with the method of this invention shows multilocular **.
It has suggested that ** is formed in ****** which is started by freeze drying, where small SSL extruded beforehand unites and which is in agreement with the increase with the average diameter of 80 to 250 nm observed.

This observation is in agreement with the already reported fusion phenomenon in the freeze drying / reconstruction process of SSL (263-268 of "Nucl.Med.Biol.22" of SUZAKKU of the

1995 issue, and a chill tap). Probably more big formation of ** will activate derivation of the VIP molecule between the distribution demanded for maintenance of small average size, and long cycle time, and inside final liposome.

Example 5 Peptide activity in the simplified liposome preparation According to this example, the simple manufacturing method of SSL combined with biological activity peptide is provided, and it acts so that consequential liposome may be approximately maintained in size of 200 nm or less. In addition, the more desirable adjustment method was examined and the effect on the peptide activity of this adjustment method was measured. Egg yolk PC, egg yolk PG, cholesterol, and PEG-DSPE are mixed by the mole ratio of 5:1:3.5:0.5 in chloroform, and this solvent was evaporated at 45 ** using the water bath. the film of lipid came out all night, and was dried, and it was prevented with the salt water which is 250microl. This mixture was stirred, and it was ultrasonicated for 5 minutes, and extruded using the RIPOSO fast (LiposoFast) device through the accumulated polycarbonate filter. Human VIP was added by the liposome whose average diameter is 300 nm or less and which was obtained as a result, and this mixture was held all night at 4 **. Free VIP was separated from VIP joint liposome using A-5 m of Biogel column, and the collected liposome was stored under 4 ** argon until it was used. The size of the liposome measured by the electronic light scattering in a certain meaning showed the 162-nm plus-or-minus 59nm average diameter. Phospholipid concentration and a VIP recovery rate were measured as mentioned above, and 50% of phospholipid in which 44% of VIP and VIP were given was found out. The mole ratio of phospholipid is 0.

It was 006.

The golden Syrian hamster of grown-up hypertension was prepared for the microcirculation of the cheek pouch by the above-mentioned technique which microscope[in the living body]-observes, is observed, and is measured, and the arterial blood pressure measured, respectively. Measurement was made by SSL without administration of VIP solution, and VIP and VIP in SSL prepared as mentioned above.

It was large, and suffusion of VIP in SSL for 7 minutes was dependent on concentration, and was seen with the increase in the arteriole diameter over a long period of time. Large vasodilatation was observed for 1 minute from the start of suffusion, and it was the maximum for 5 minutes of the first stage. The arteriole diameter usually returned to the level in 8 minutes after the end of suffusion. VIP of solution and empty SSL were completely ineffective.

VIP in SSL also actualized the sharp fall of the blood pressure of a blood vessel, and the optimum was observed for 30 minutes from the start of suffusion. Blood pressure was maintained by the slight lowness in the observation period of 6 hours. VIP of solution and empty SSL were completely ineffective like the above.

As for this result, drying/rehydration step described in Example 3 showed that it was not

necessarily required for the composition of activity liposome preparation. The liposome adjusted to the more important thing by this method maintained the average diameter of 200 nm or less, and even though it was not higher than the liposome preparation of the gap to be described in Example 3, equivalent VIP activity was maintained. VIP obtained from this adjustment method as further advantage: When the ratio of phospholipid compares with the method of Example 3, it is high (0.006 to 0.004).

Example 6 SSL containing SSLVIP in sound wave reflective evaluation was prepared, and imaging using the following sound wave reflective measurement was presented.

The liposome prepared as described by Example 3 was moved to the liquid scintillation bottle, and was imagined by a 20-MHz high frequency blood vessel ultrasonic wave (IVUS) imaging catheter (Sunnyvale of Boston Scientific Corp., CA). This IVUS catheter had the cap of a bottle let it pass, and was fixed. The instrument set for a profit, zoom, compression, and a refusal level was optimized at the time of an experiment start, and was uniformly held about all the samples. The image was recorded on 1/2-inch VHS videotape by real time for next playback and image analysis. The relative echogenicity (clear luminosity) of liposome formulation was objectively assessed by the computer support densimeter. The process contained acquisition, a previous process, the automated liposome identification, and a gray scale fixed quantity. An image process and analysis were carried out by the image prop lath software (Ver1.0, media cyber-NETIKUSU, Silver Spring, MD) started on a computer (486CPU, 66 MHz) for exclusive use. The IVUS image selected at random was acquired from the videotape of each liposome formulation. The image was digitized by 640x480 picture-element-region resolution (approximately 0.045 mm/(pixel)) and resolution with a size of 8 bits (256 levels). All the analyzed IVUS data was collected on the acquisition level of the fixed instrument. And distribution of the gray scale value within an image was adjusted so that the total range of the possible gray level which used the linear transformation algorithm might be covered (that is, the dynamic range was maximized). The brightness of the image was expanded and contracted subjectively, the feature of reference was common with all the images, and the gray scale value over all the images was maintained uniformly. And the automated liposome detection routine was started for identification of the liposome suspended in the solvent in the annular region of the relation which constant-distance-separated radially and was set from the image catheter. the automated liposome detection routine identified all the analysis endocyclic bright objects which has a bigger gray scale level than 29, a circle ratio (namely, ratio of a maximum diameter and a minimum diameter) of 2.5 less than, and the size of 4 pixels or more. This procedure eliminated all the image artefacts substantially from detection algorithms. Therefore, the identified object is considered to be "liposome." The outline was drawn by the computer program and each liposome was counted. And since the echogenicity of the liposome formulation which was computed and was given was characterized, the gray scale of

each value of all the pixels and the average of size which were identified "liposome" in the given image were used. These experimental results show that sound wave reflection of VIP liposome preparation has the gray scale (it can set to the gray scale from 0 to 255 which is pure black and which is pure white) of 119. Big liposome is characterized by sound wave reflection of about 110-120, and liposome provided with a contrast medium like Albunex (registered trademark) has sound wave reflection of about 110-120 rather than manufactured using the freeze drying process stated to PCT gazette WO93/20802. That is, this invention provides the liposome of a small diameter, maintaining the auditory image property.
By indication in the example illustrated above, it is foreseen that improvement and change of many of this inventions take place to a person skilled in the art. Therefore, only limitation as shown in the attachment claim should be positioned in this invention.

[Translation done.]